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14. ABSTRACT
Stathmin is a microtubule destabilizing protein that interacts with two tubulin heterodimers to form a ternary stathmin-tubulin complex. This interaction is critical for the dynamic regulation of interphase & mitotic microtubules. Here, we asked whether intracellular delivery of stathmin-like fusion peptide(s) could prevent the normal association of stathmin with tubulin and mediate an anti-proliferative effect in in vitro models of human breast cancer. Evaluation of intracellular uptake of the peptide(s) demonstrated that breast cancer cells could effectively take up the designed peptides. Proliferation assays showed significant growth inhibitory effects in breast cancer cells exposed to wild type or mutant peptides compared to cells exposed to a control peptide. Furthermore, immunofluorescence analysis of breast cancer cells exposed to the designed peptides showed marked inhibition of microtubule polymerization. This suggests that the observed antiproliferative effect is likely a result of inhibition of microtubule assembly. Our current studies are aimed towards evaluating the biologic effects of the fusion peptide(s) in the absence & presence of vinblastine to determine if the fusion peptide(s) interact synergistically with vinblastine in breast cancer cells. These studies should provide the proof-of-principle that peptide(s) could be used to modulate stathmin function and inhibit malignant proliferation of breast cancer cells.

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A. INTRODUCTION

Breast cancer is one of the most frequent malignancies and is the major leading cause of cancer-related deaths in women in the U.S. When local therapies for breast cancer fail and the disease progresses, systemic estrogen ablation therapy, with or without chemotherapy, can lead to tumor regression. However, the disease inevitably progresses to an estrogen-independent state that becomes resistant to hormonal therapy and chemotherapy. In this advanced stage, there are currently no curative therapies as such patients almost always die from their metastatic disease. Thus, there is clearly a pressing need for the development of alternative strategies for this devastating malignancy.

Stathmin is the founding member of a family of microtubule destabilizing proteins that regulate the polymerization & depolymerization of the microtubules through its cell cycle specific phosphorylation [1-4]. Numerous studies, including our own, first demonstrated that stathmin plays an important role in the regulation of cell proliferation [2, 4-6]. This was followed by the identification of stathmin as a major regulator of the dynamics of microtubules that make up the mitotic spindle [7]. Thus, stathmin is one of the key regulators of the microtubule cytoskeleton and the mitotic spindle [1, 2, 4, 7, 8]. Stathmin promotes microtubule depolymerization either by increasing the rate of microtubule catastrophe or by sequestering tubulin thus depleting the pool of tubulin available for polymerization [1, 7, 9-11]. Both activities of stathmin are modulated by the interaction of stathmin to two tubulin heterodimers to form a ternary (T2S) stathmin-tubulin complex that directly contributes to the dynamic regulation of microtubules during cell cycle progression [7, 9, 10, 12]. In addition to its well-documented role in cellular proliferation, stathmin is also expressed at high levels in a wide variety of human malignancies including breast cancer. Of particular significance, the high level of stathmin expression has been shown to correlate with the malignant behavior of breast cancer cells, proliferation cell nuclear antigen (PCNA) expression, large tumor size, high tumor stage and poor prognosis [13-15]. Thus, the level of stathmin expression serves as an important prognostic marker that predicts survival in breast cancer and provides an attractive target for breast cancer therapy.

Recently, numerous short peptides from the N-terminal regions of different members of stathmin family were shown to impede tubulin polymerization with different efficiencies [16]. The inhibition of tubulin polymerization was a result of direct stoichiometric interaction of the peptide with tubulin to form a peptide/tubulin complex [16]. The most efficient of these peptides, I19L, was a 19-residue peptide that covers the N-terminal domain of stathmin (residue 6-24) [16]. The ability of I19L to impede tubulin polymerization was also found to be markedly reduced by Ser-16 phosphorylation [16]. Thus, this peptide possesses an autonomous anti-MT activity [16]. A major goal of our study is to test the hypothesis whether intracellular delivery of stathmin-like peptide(s) would interfere with the normal association of stathmin with tubulin and inhibit the malignant proliferation of breast cancer cells by disrupting microtubule assembly and the spindle apparatus. Unlike taxanes that stabilize microtubules, vinca alkaloids destabilize microtubules. The combination of stathmin-based peptide(s) with vinca alkaloids is particularly attractive since both agents inhibit microtubule assembly. Thus, a secondary hypothesis is whether stathmin-like peptide(s) would interact synergistically with vinblastine in breast cancer cells *in vitro*.

B. BODY

In our grant application entitled " Novel antimicrotubule agents for breast cancer", we had proposed three specific aims: 1) To test the ability of stathmin-like peptide(s) to bind tubulin and impede microtubule assembly in *in vitro* models of breast cancer cell lines; 2) To test the biologic effects of the peptides on proliferation, clonogenicity & apoptosis in different breast cancer cell lines *in vitro*; & 3) To determine if the combination of stathmin-like peptide(s) & anti-microtubule drugs like vinblastine would result in synergistic anti-tumor effects in breast cancer cell lines. In this final report, we will summarize all the studies performed during the entire grant period to achieve the above specific goals.

1. DESIGN OF THE STATHMIN-LIKE PEPTIDES:

The peptide(s) that we designed consisted of the same 19 residues (residues 6-24) from the N-terminal domain of stathmin [16]. This peptide was fused to a TAT transduction domain (residues 48-60) [17], that served as a carrier to facilitate entry into breast cancer cells. In order to be able to track this fusion peptide intracellularly, we also included a hemagglutinin (HA) epitope tag. This peptide was named wild-type stathmin peptide (W-SP). Since W-SP could be phosphorylated & inactivated intracellularly by $p34^{cdc2}$ kinase [5, 16], we also made another peptide in which Ser-16 was mutated to an alanine to prevent its inactivation by phosphorylation. Previous studies had shown that this substitution results in a more potent form of stathmin that cannot be inactivated by phosphorylation [16]. The resulting mutant peptide was named W-SaP. As a control, we generated a similar peptide in which the TAT and HA sequences were identical to W-SP but the stathmin-like domain was totally scrambled (Sc-P). All peptides were chemically synthesized at >95% purity at GenWay Biotech, Inc.

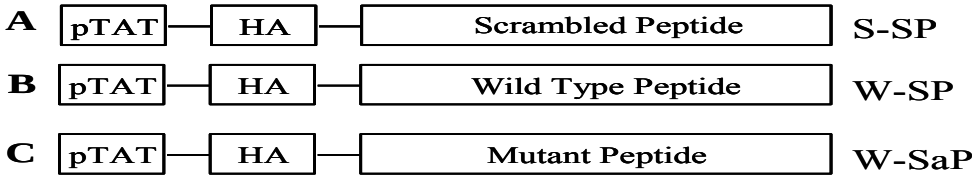

Fig.1 Schematic illustration of different fusion peptides. The domains are labeled. A. Design of scrambled peptide (Sc-P). B. Design of wild-type stathmin peptide (W-SP). C. Design of mutant stathmin peptide (W-SaP). The single difference between the two peptides, W-SP & W-SaP, is the substitution of the serine-16 to alanine to prevent inactivation of the peptide by phosphorylation.

2. INTRACELLULAR TRAFFICKING OF THE DESIGNED PEPTIDES:

To determine whether the designed peptide(s) are taken up intracellularly, we first tracked the intracellular uptake of the peptide(s) in breast cancer cells exposed to the different peptide(s) by immunostaining with Alexa Fluor 488 conjugated anti-HA antibody. Since stathmin's role in mitosis starts by promoting the depolymerization of interphase microtubules before the breakdown of the nuclear membrane [1, 2], we had used a mutant TAT sequence that was previously shown to

maintain the cargo peptide in the cytoplasm [18]. Fig. 2 shows representative images of T47D breast cancer cells exposed to different peptides. The presence of the peptide(s) was detected primarily in the cytoplasm of breast cancer cells as expected (Fig.2). Furthermore, nearly 85% **of** the breast cancer cells were positive for HA staining suggesting that a vast majority of cells had taken up the peptide(s). Similar findings were observed in other breast cancer cells including SKBR-2 and MCF-7 breast cancer cell lines.

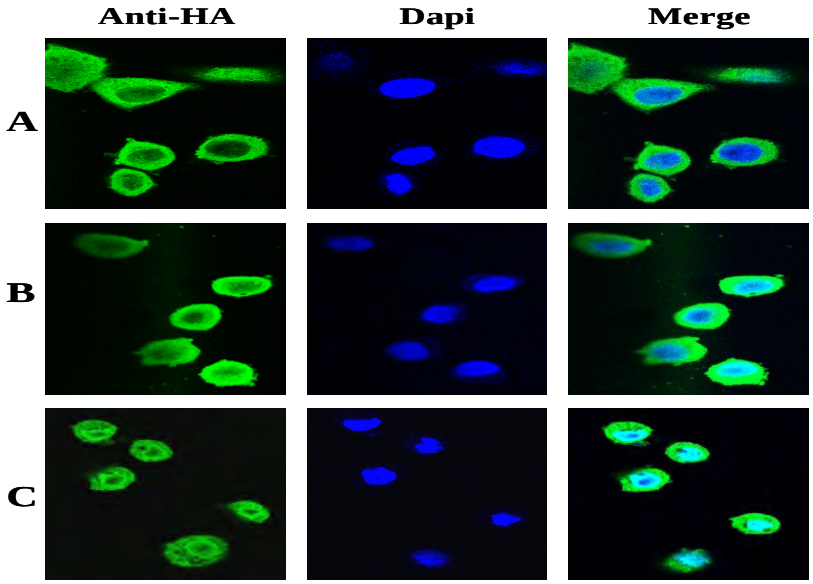

Fig.2 Intracellular localization of the designed peptide(s) by confocal microscopy. These are representative images of T47D breast cancer cells that were exposed to different peptide(s) overnight. Next day, the cells were fixed, permeabilized and stained with Alexa Fluor 488 conjugated anti-HA antibody. A, B & C are representative images of T47D cells exposed to Sc-P, W-SP and W-SaP peptides respectively. The left panel shows images stained with Alexa Fluor 488 conjugated anti-HA antibody, the middle panel shows images stained with DAPI and the right panel shows images in which the Alexa and DAPI staining were merged.

3. EFFECTS OF THE DESIGNED PEPTIDES ON THE PROLIFERATION OF BREAST CANCER CELLS:

Since this peptide mimics the tubulin sequestering activity of the entire stathmin molecule, we predicted that its presence in cancer cells would promote microtubule depolymerization as was previously observed in stathmin over-expressing cells [19]. This in turn should prevent the formation of a functional mitotic spindle and mediate an anti-proliferative effect. Thus, we examined the effects of the designed peptides on the rate of proliferation of breast cancer cells using a non-radioactive MTT (Methylthiazoletetrazolium) assay. This assay is based on the conversion of tetrazolium salt into a blue formazon dye [20]. Growth curves of breast cancer cell lines exposed to different peptides are shown in Fig.3. When breast cancer cells were exposed to control Sc-P at different concentrations, the rate of proliferation was essentially unchanged and the cells continued to proliferate like the untreated cells (Fig.3, left panel). Exposure of breast cancer

cells to wild-type stathmin peptide, W-SP, at a lower concentration of 2 uM showed a moderate decrease in the rate of proliferation in the different cell lines (Fig.3, middle panel). However, when the concentration of the W-SP was increased to 5 uM, the rate of proliferation was markedly decreased in all three cell lines as shown in Fig.3 (middle panel). Interestingly, when breast cancer cells were exposed to the mutant peptide, W-SaP, at the same concentrations (2 and 5 uM), the growth inhibitory effects were much more pronounced even at a lower concentration of 2 uM and there was a complete cessation of growth at a concentration of 5 uM (Fig.3, right panel). In other words, exposure of breast cancer cells to low concentrations (2 uM) of wild-type stathmin peptide moderately inhibited proliferation, while exposure to the same concentration of mutant peptide was sufficient to result in a near complete suppression of growth. Thus, this data demonstrates that both, wild-type & mutant peptides can inhibit the growth of breast cancer cells *in vitro*. However, the mutant peptide mediates a more profound anti-proliferative effect. This is not surprising since the mutant peptide cannot be inactivated by phosphorylation as discussed above.

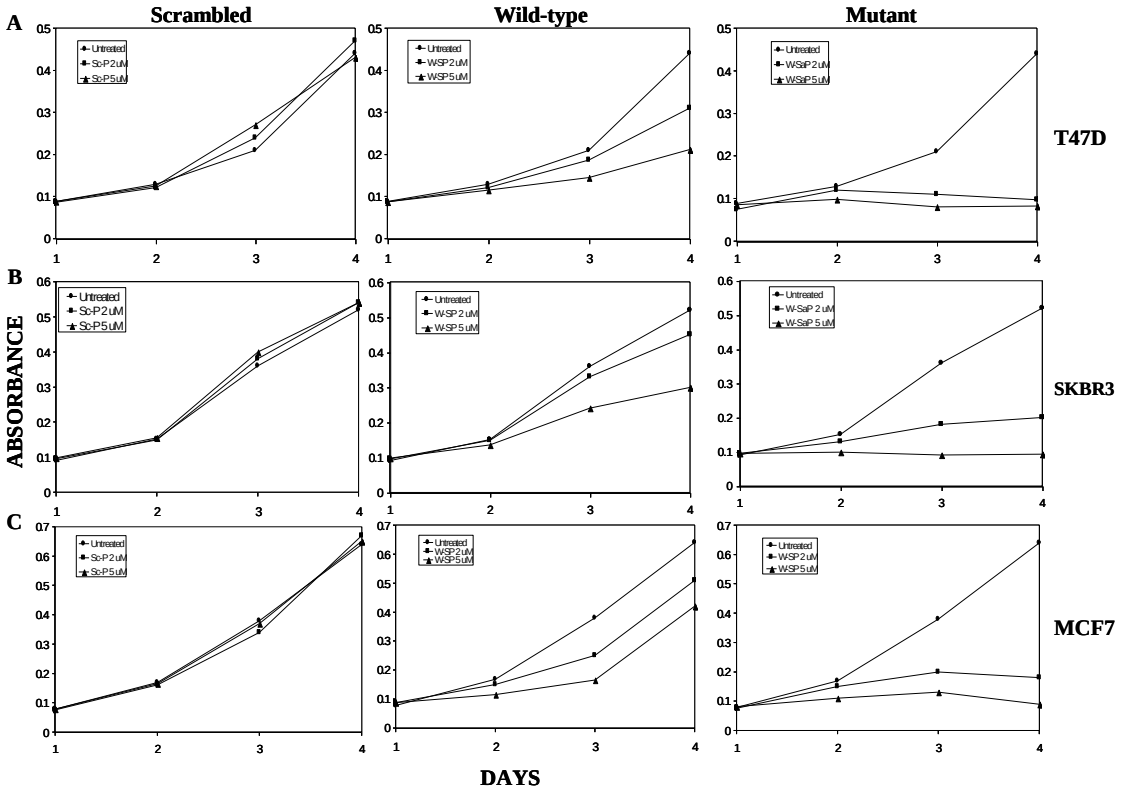

Fig.3 Effects of the designed peptides on the rate of proliferation of breast cancer cell lines. Breast cancer cells were plated equally at a density of 1000 cells/well in 96 well plates and exposed to either Sc-P, W-SP or W-SaP in triplicates for 4 days. The cells were stained with MTT for 3 hours and absorbance was measured at 570 nm in a microplate reader. A. Growth rates of untreated T47D cells or T47D cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as indicated. B. Growth rates of untreated SKBR3 cells or SKBR3 cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as indicated. C. Growth rates of untreated MCF7 cells or

MCF7 cells exposed to Sc-P, W-SP or W-SaP peptide at different concentrations as indicated. The growth curves were generated by plotting the means of triplicate absorbance measurements based on cellular conversion of tetrazolium salt.

4. EVALUATION OF MICROTUBULE ORGANIZATION:

Stathmin is one of the key regulators of the microtubule cytoskeleton that regulate the dynamics of microtubules by promoting microtubule depolymerization. Since the N-terminal stathmin peptide was previously shown to impede microtubule assembly in *in vitro* polymerization assays [21], we asked whether intracellular delivery of the designed peptides would result in destabilization of microtubules in breast cancer cells. Thus, we examined the effects of different peptides on the microtubule cytoskeleton in breast cancer cells by immunofluorescence analysis of microtubules (Fig. 4) as we had previously described [4, 22]. Exposure of breast cancer cells to control Sc-P showed normal organization of microtubule network (Fig.4A). In contrast, when cells were exposed to W-SP or W-SaP, there was a reduction in microtubule density (Fig.4B & C) compared to control cells (Fig.4A). However, the reduction in microtubule density was much greater in cells exposed to mutant W-SaP (Fig.4C) than in cells exposed to wild type W-SP (Fig.4B) as expected. A similar decrease in microtubule density was also observed in SKBR3 and MCF7 breast cancer cells. This data demonstrates that the designed peptides can inhibit microtubule polymerization in a cellular environment. Thus, the pronounced anti-proliferative effect observed with mutant peptide is likely a result of inhibition of microtubule assembly.

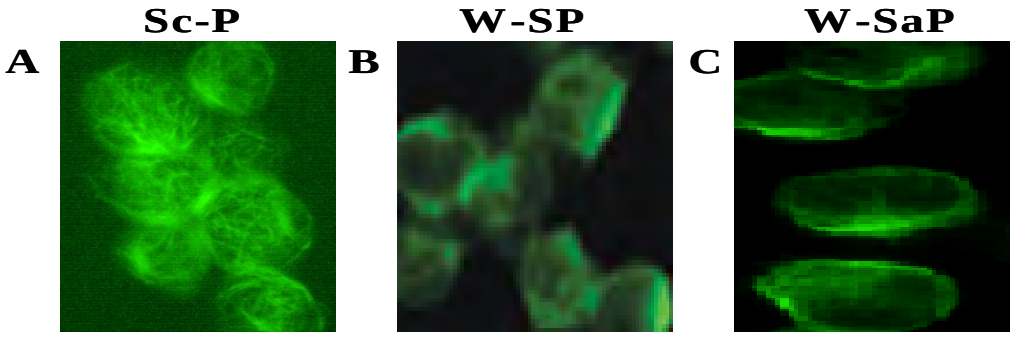

Fig.4 Effects of the designed peptides on the microtubule cytoskeleton of breast cancer cells. These are representative images of T47D cells exposed to different peptides. Cells plated in chamber slides were exposed to either Sc-P, W-SP or W-SaP overnight. Next day, the cells were fixed & stained with fluorescein conjugated anti-tubulin antibody. The images were captured at 100x under oil immersion. A, B & C are representative photograph of MT network in cells treated with Sc-P, W-SP & W-SaP respectively.

5. EFFECTS OF THE DESINGED PEPTIDES ON TUBULIN POLYMERIZATION:

We had evaluated the levels of polymerized and unpolymerized tubulin in cells exposed to control, wild type or mutant stathmin-like peptides using a sensitive biochemical assay that we described in our previous reports (4, 22). This assay is based on the isolation of in vivo assembled microtubules in their polymerized state and the unpolymerized tubulin as a soluble protein (4, 22). The two forms of tubulin were separated by ultracentrifugation and the levels of polymerized and soluble tubulin

were quantified by immunoblotting with an anti-tubulin antibody. Immunoblot analysis showed a 1.2 fold decrease in the ratio of polymerized to soluble tubulin in cells exposed to wild-type peptide relative to control cells. The ratio of polymerized to soluble tubulin was further decreased to 2.3 fold in cells exposed to mutant peptide relative to control cells. These findings are compatible with the observed reduction in microtubule density in the above experiment. Thus, this data suggests that the designed peptides can interfere with the polymerization of the microtubules.

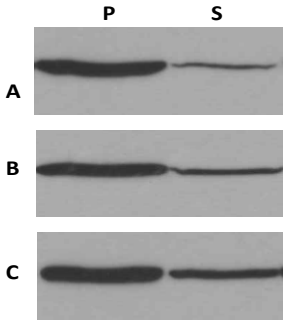

Fig.5 Effects of the designed peptide on tubulin polymerization. (A) Representative autoradiograph of western blots of polymerized (P) and soluble (S) forms of tubulin derived from cells exposed to control Sc-P. (B) Representative autoradiograph of western blots of polymerized (P) and soluble (S) forms of tubulin derived from cells exposed to W-SP. (C) Representative autoradiograph of western blots of polymerized (P) and soluble (S) forms of tubulin derived from cells exposed to control W-SaP.

6. EVALUATION OF PEPTIDE-TUBULIN INTERACTION:

The intracellular interaction of the designed peptide(s) with tubulin was evaluated using a co-immunoprecipitation assay using anti-tubulin antibody & protein A agarose beads followed by western blotting with anti-HA antibody. Fig. 6 shows western blots on immunoprecipitates derived from breast cancer cells exposed to either Sc-P, W-SP or W-SaP. When the blot was probed with anti-tubulin antibody, tubulin protein was detected in lysates prepared from cells exposed to Sc-P, W-SP & W-SaP (Fig.6B, lanes 2, 3 &4) but not in lysate incubated with preimmune serume as expected (Fig.6, lane 1). We predicted that if the peptide interacts with tubulin, then the anti-HA antibody should be able to pull down the peptide-tubulin complex. Thus, when the same blot was probed with anti-HA antibody, a strong signal was detected in lysates prepared from cells exposed to W-SP & W-SaP peptides (Fig.6B, lanes 3 &4). In contrast, no signal was detected in lysate incubated with preimmune serum as expected (Fig.6B, lane 1). Similarly, no signal was detected in lysate prepared from cells exposed to Sc-P, since Sc-P is not expected to interact with tubulin (Fig.6B, lane 2). This data demonstrates that the designed peptide(s) are capable of interacting with tubulin intracellularly.

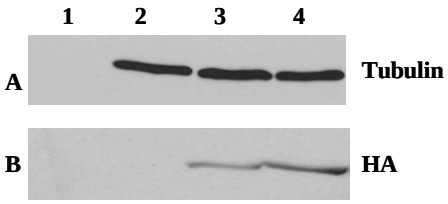

Fig.6 Co-immunoprecipitation of the peptide-tubulin complex in breast cancer cells. Cell lysates prepared from equal number of T47D cells were exposed to either Sc-P, W-SP or W-SaP overnight. Tubulin was immunoprecipated by incubation with anti-tubulin antibody at 4^oC overnight. As a control, lysate prepared from cells was incubated with preimmune serum at 4^oC overnight. Next day, the immunecomplexes were adsorbed on protein A agarose beads at 4^oC for 2 hrs. The immuno-complexes were collected by centrifugation, washed and then subjected to SDS-PAGE followed by western blotting using an anti-tubulin or anti-HA antibody. The bands were detected by chemiluminescence. A. Western blot probed with anti-tubulin antibody. B. Western blot probed with anti-HA antibody. Lanes 1 represents lysate incubated with preimmune serum. Lane 2, 3 & 4 represents lysate prepared from cells exposed to Sc-P, W-SP & W-SaP respectively.

6. EFFECTS OF COMBINATION OF THE DESIGNED PEPTIDES AND ANTI-MICROTUBULE AGENT ON THE MALIGNANT PHENOTYPE OF BREAST CANCER CELLS:

We next evaluated the effects of the designed designed peptides on the malignant phenotype of breast cancer cells in combination with anti-microtubule agents like vinblastine. We first evaluated the effects of the combination of the two agents on the proliferation of breast cancer cells. We used low concentration of the peptides (2 uM) and low concentration of vinblastine below IC50 in all the combination studies described below to determine if the effects of the designed peptides could be further enhanced by addition of vinblastine. Fig. 7 illustrates the effects of combination of different concentrations of vinblastine and the peptides on the rate of proliferation of cells. The rate of proliferation of untreated cells or cells exposed to control peptide (Sc-P) was not affected by low concentration of vinblastine (2 nM) and was modestly inhibited at a concentration of 5 nM. When cells were exposed to W-SP and vinblastine, the rate of proliferation was further decreased at a concentration of 2 nM vinblastine and nearly suppressed at a concentration of 5 nM vinblastine. In contrast, cells exposed to W-SaP showed complete cessation of growth when combined with 2 nM or 5 nM vinblastine. Thus, exposure to mutant peptide seemed to result in greater sensitization of breast cancer cells to the growth inhibitory effects of vinblastine.

We also investigated the ability of the designed peptides on the clonogenic potential of breast cancer cells using a soft agar assay. The soft agar assay for colony formation is an anchorage independent growth assay in soft agar, which is considered the most stringent assay for detecting malignant transformation of cells. For this assay, cells were pretreated with the peptides in the presence and absence of vinblastine for 4 days and were cultured in soft agar medium for two weeks. After two weeks incubation, the colonies that formed were quantified (Fig.8). Cells exposed to either W-SP or W-SaP showed a 22% reduction in the colony formation respectively (Fig. 8A). When cells were exposed to either W-SP or W-SaP in the presence of vinblastine, the clonogenicity was further reduced to 35% and 72% respectively. Thus, this data demonstrates that the peptides can inhibit anchorage independent growth in soft agar, which can be markedly enhanced by vinblastine.

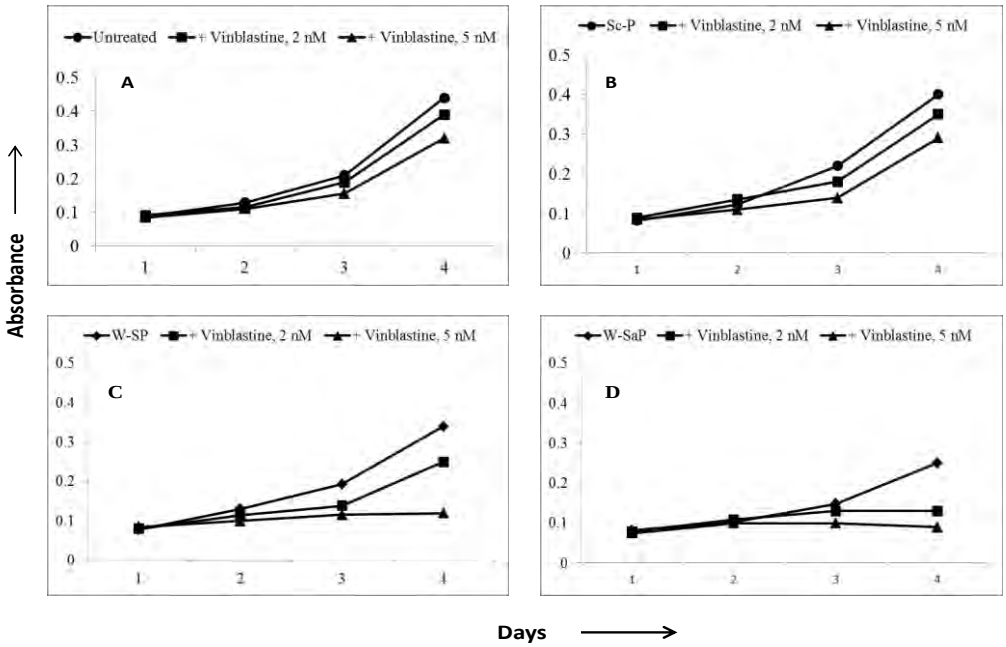

Fig. 7 Effects of combination of vinblastine and stathmin-like peptides on the rate of proliferation of breast cancer cells. Equal number of T47D cells were exposed to low concentration of the peptides (2 uM) and low concentrations of vinblastine (2 & 5 nM) for 5 days. The cells were stained with MTT for 3 hours and absorbance was measured at 570 nm in a microplate reader. (A) Growth curves of untreated T47D cells or cells exposed to different concentrations of vinblastine as indicated. (B) Growth curves of T47D cells exposed to low concentration of the Sc-P (2 uM) and different concentrations of vinblastine as indicated. (C) Growth curves of T47D cells exposed to low concentration of the W-SP (2 uM) and different concentrations of vinblastine as indicated. (D) Growth curves of T47D cells exposed to low concentration of the W-SaP (2 uM) and different concentrations of vinblastine as indicated.

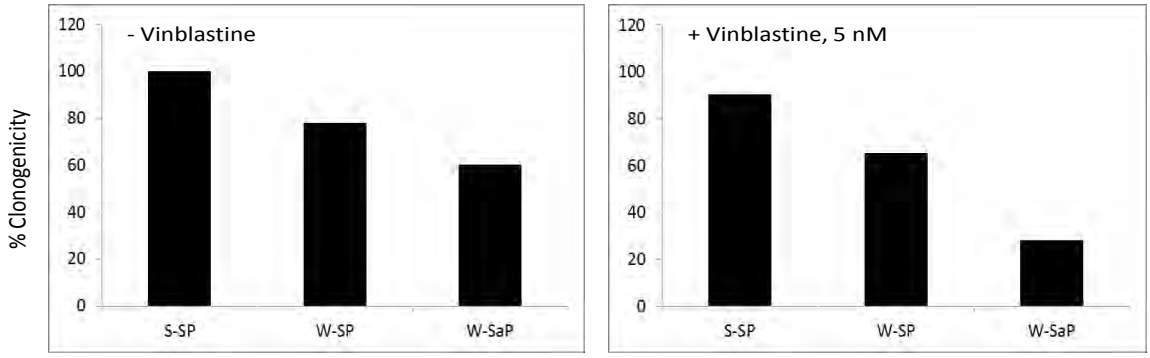

Fig. 8 Effects of stathmin-like peptides on colony formation in soft agar. T47D **c**ells were pretreated with the peptides in the presence and absence of vinblastine for 4 days and were cultured in soft agar medium for two weeks. After two weeks incubation, the colonies that formed were quantified. (A) Bar graph showing clonogenicity of Sc-P, W-SP or W-SaP in the absence of vinblastine as indicated. (B) Bar graph showing clonogenicity of Sc-P, W-SP or W-SaP in the presence of vinblastine as indicated.

We next analyzed the ability of the designed peptides to induce apoptosis either as a single agent or in combination with vinblastine using a TUNEL assay. Fig.9 illustrates the effects of the peptides in combination with vinblastine on apoptosis. TUNEL analysis of control cells showed essentially no apoptosis. When the cells were exposed to stathmin-like peptides, there was a 13% and 24% increase in the fraction apoptosis in wild type and mutant peptide treated cells respectively (Fig.9A). However, when cells were exposed to a combination of stathmin-like peptides and vinblastine, the fraction of TUNEL positive cells was markedly increased to 60% and 84% in wild type and mutant peptide treated cells respectively (Fig.9A). Representative images of cells treated with mutant peptide showing TUNEL positivity in a vast majority of cells is shown in Fig. 9B.

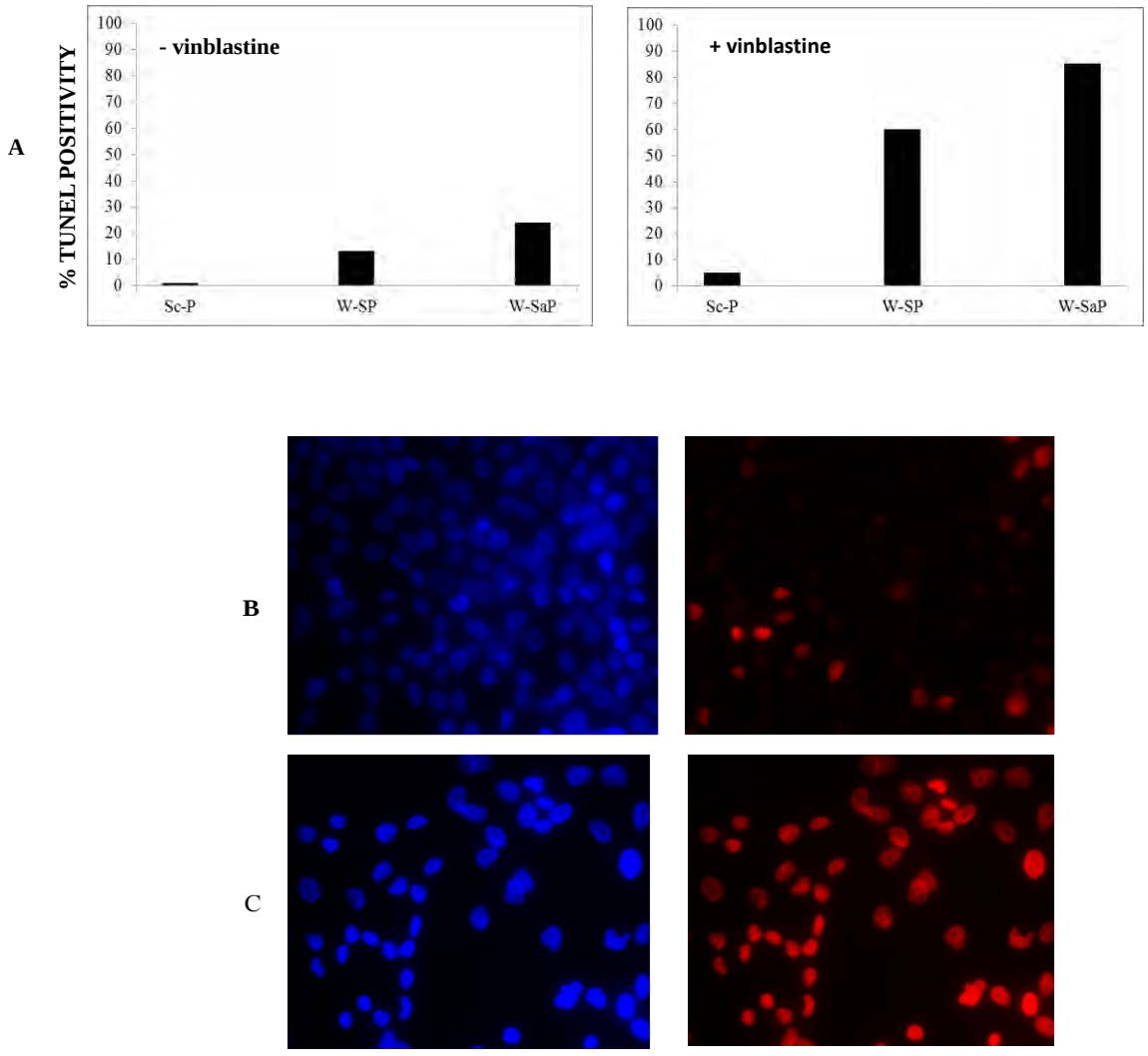

Fig.9 Effects of stathmin-like peptides and vinblastine on apoptosis. Equal number of T47D cells was exposed to Sc-P, W-SP or W-SaP in chamber slides for 5 days. The cells were fixed in 2% paraformaldehyde and subjected to TUNEL (Terminal Deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling) assay. The fraction of TUNEL positive cells was assessed by microscopy. (A) Bar graphs showing the percentage of TUNEL positive cells in cells exposed to Sc-P, W-SP or W-SaP in the absence or presence of vinblastine as indicated. (B) Representative images

showing TUNEL positivity in cells exposed to W-SaP in the absence of vinblastine for 5 days. (B) Representative images showing TUNEL positivity in cells exposed to W-SaP in the presence of vinblastine (5 nM) for 5 days. The left panel in blue shows cells stained with dapi. The right panel in red shows cells subjected to TUNEL reaction.

C. KEY RESEARCH ACCOMPLISHMENTS

(i) The experiments described above demonstrate that breast cancer cells can effectively take up the designed stathmin-like peptides.
(ii) We also demonstrate that the peptides can bind tubulin and can interfere with the assembly of microtubules.
(iii) We also demonstrate that intracellular delivery of stathmin-like peptides can inhibit the proliferation of breast cancer cells, *in vitro*. Inhibition of cell proliferation by the peptides was markedly enhanced by vinblastine.
(iv) We also demonstrate that the peptides can inhibit colony formation, which was markedly enhanced by vinblastine.
(v) We also show that the stathmin-like peptides are capable of inducing apoptosis, which is significantly increased by vinblastine.

D. REPORTABLE OUTCOMES

Some of the research studies summarized in this report were disseminated at the Era of Hope, Department of Defense Breast Cancer meeting (Abstract # P23-13, 2011 Proceedings). Additional submission of an abstract at the AACR meeting and a manuscript describing this work is being planned.

E. CONCLUSIONS

We conclude that the designed stathmin-like peptides are capable of inhibiting the growth of breast cancer cells *in vitro.* Moreover, the combination of the designed peptides and vinblastine greatly enhanced the anti-proliferative and anti-tumor activity in breast cancer cells in culture. We hope to extend these studies in animal models of breast cancer. The success of these studies may set the stage in the future as a step towards translating this knowledge into an effective therapeutic approach in patients with breast cancer.

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